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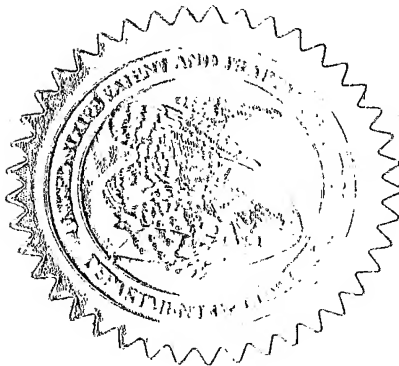
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COMMISSIONER OF PATENTS AND TRADEMARKS**



*L. Edelen*

**L. EDELEN  
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13281 U.S. PTO

Page 1 of 1U.S. PATENT AND TRADEMARK OFFICE  
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**TITLE OF THE INVENTION (280 characters max)**

A METHOD FOR DETECTING TELOMERASE

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**ENCLOSED APPLICATION PARTS (check all that apply)**

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Respectfully submitted,

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## A METHOD FOR DETECTING TELOMERASE

### FIELD OF THE INVENTION

The present invention is generally in the field of detection of an analyte in a sample, and more specifically detection of an analyte indicative of cancer.

### BACKGROUND OF THE INVENTION

5       The preparation of DNA-based enzymes attracts substantial research efforts directed to the development of novel biocatalysts.<sup>1,2</sup> Many different nucleic acids were employed as catalysts for different chemical transformations such as cleavage of RNA or DNA phosphoesters,<sup>3</sup> porphyrin metallation,<sup>4</sup> and DNA ligation.<sup>5</sup> An interesting example of a catalytic DNA is a single-stranded guanine-rich nucleic  
10 acid (aptamer) that upon complexation with hemin revealed peroxidase activity<sup>6</sup> (the H<sub>2</sub>O<sub>2</sub> mediated oxidation of 2,2'-azinobis(3-ethylbenzothiozoline)-6-sulfonic acid, ABTS). It was suggested that the intercalation of the hemin into the guanine-quadruplex docked layers of the nucleic acid leads to the biocatalytic structure.

Enzymes are often employed as biocatalysts for the amplified detection of  
15 DNA. Horseradish peroxidase (HRP) was used as biocatalytic label for the amperometric detection of DNA.<sup>7</sup> The biocatalyzed precipitation of an insoluble product on electrode surfaces was reported as a means for the amplified electrochemical sensing of DNA,<sup>8,9</sup> and the generation of redox-active DNA replica and the secondary activation of bioelectrocatalytic processes was used for  
20 the amplified amperometric analysis of DNA.<sup>10</sup> Recently, the analysis of DNA by the HRP-mediated generation of chemiluminescence was reported.<sup>11</sup> In this system, doxorubicin was intercalated into double stranded DNA, and the doxorubicin-mediated electrocatalyzed generation of H<sub>2</sub>O<sub>2</sub> allowed the generation of chemiluminescence in the presence of luminol and HRP.



The chromosomes are protected by nucleic acids of constant repeats termed telomeres.<sup>13</sup> The gradual erosion of the telomere units during cell proliferation provides a cellular signal for terminating the cell cycle. In certain cells there is accumulation of the ribonucleoprotein telomerase that incorporates the telomere units into the chromosome ends, and this turns the cells into immortal entities.<sup>14</sup> Indeed, in over 95 % of the different cancer or malignant cells, elevated amounts of telomerase were detected,<sup>15</sup> and the monitoring of telomerase activity in cells is promising for cancer diagnostics.<sup>16</sup> Several analytical procedures for the determination of telomerase activity were developed and these include the TRAP method<sup>17</sup> (telomeric repeat amplification protocol), the fluorescence detection of telomerase activity,<sup>18</sup> or the recently reported<sup>19</sup> optical detection of telomerase using CdSe/ZnS quantum dots.

## SUMMARY OF THE INVENTION

The present invention is based on the realization that catalytic polynucleotides, in particular DNAzymes, are capable of detecting the presence of telomerase activity in a sample, by detecting the formation of telomerase-catalyzed repeats sequences, or a pre-provided primer.

This realization paves the way for the production of a method for detection of cancer, by the detection of said telomerase activity.

The detection of the activity of the catalytic polynucleotide may be achieved by one of two manners. In accordance with one embodiment, the detection is achieved by surface detection of products produced by immobilized catalytic polynucleotides. By another embodiment, the detection is by solely soluble components ("beams") which in the presence of the repeat sequence produced by the telomerase reverts from a pre-catalytic to a catalytic form..

By the first "surface detection" aspect, the present invention concerns a method for detection of telomerase activity in a sample the method comprising:



- a) providing a primer for telomerase activity immobilized on a solid surface;
- b) contacting the sample with the immobilized primer in the presence of dNTP's, under conditions enabling formation of a telomerase repeat sequence;
- 5 c) adding a catalytic polynucleotide, attached to a sequence complementary to the telomerase repeat unit;
- d) removing unbound catalytic polynucleotides;
- e) providing substrates for catalytic polynucleotides; and
- 10 f) detecting the presence of catalytic products of catalytic polynucleotides, said presence indicating the presence of telomerase activity in the sample.

The term "*sample*" refers to any liquid sample suspected of having active telomerase therein, such as body fluids (blood, plasma, urine, cerebrospinal fluid, saliva, semen) or medium in which cells, suspected of having telomerase activity, have been incubated or lysed.

The "primer" is any primer on which telomerase elongation activity may take place and is particular is:

20

5' TTTTTAATCCGTCGAGCAGAGTT

The primer may be immobilized on any surface such as walls of a vessel, beads, etc. by any means known for immobilization of nucleic acid sequences.

The term "telomerase repeat sequence" refers to the minimal unit which the telomerase add to the primer. This term can refer to several such units connected to each other in tandem.

The term "*catalytic polynucleotides*" refer to any nucleic acid sequence, which has catalytic activity. The catalytic polynucleotides may be Ribozymes or



DNAzymes, and in a preferred embodiment may be the G-rich nucleic acid sequence that binds hemin and thus has a peroxidase activity.

The catalytic polynucleotide may be, *a priori* (for example already a complex of DNAzyme and hemin), active, or may be activated after addition of an appropriate co-factor (hemin for DNAzyme,  $Mg^{++}$  for ribozyme).

The catalytic polynucleotide is attached (by covalent binding or any other binding) to a sequence that is complementary to some of the repeats formed by the telomerase activity. It should be noted that once a repeat is formed, several catalytic polynucleotides may hybridize therewith, thus significantly increasing the amount of the detectable label the sensitivity of the detection method.

The non-bound catalytic nucleotides are removed, for example by washing. The bound catalytic nucleotides are then detected by their catalytic activity, which may be any catalytic activity known to be catalyzed by catalytic nucleotides to produce a detectable catalytic product.

The activity may be cleavage splicing, rearrangement, phosphorylation (known in ribozymes). More specifically the activity may be peroxidase activity known by DNAzyme (with hemin).

By one option the peroxidase activity may be the  $H_2O_2$  mediated oxidation of 2-2' azinobis (3-ethylbenzothiozoline)-6-sulfonic acid (ABTS).

By a preferred embodiment the peroxidase activity is the generation of chemiluminescence in the presence of  $H_2O_2$  and luminal, and the detection is of chemiluminescence.

Detection of said chemiluminescence is indicative to the presence of immobilized catalytic polynucleotides and hence the presence of telomerase generated repeat units, indicating the presence of catalytically active telomerase in the sample.

By another embodiment, the "*soluble component element*" (also termed "*beacon*") the present invention concerns a method for detection of the presence of catalytically active telomerase in a sample, the method comprising:



- (a) providing a pre-catalytic complex comprising a catalytic nucleic acid sequence attached to an inhibitory sequence, said inhibitory sequence being complementary to a telomerase repeat sequence, the inhibitory sequence, in the absence of the telomerase-repeat sequence, inhibiting the catalytic activity of the catalytic nucleic acid sequence while in the pre-catalytic complex, the complex further comprising a primer for telomerase elongation;
- (b) contacting the pre-catalytic complex with the sample in the presence of dNTPs and under conditions enabling primer elongation by telomerase;
- (c) providing substrates for catalytic polynucleotides;
- (d) detecting the presence of catalytic products of the catalytic polynucleotides, detection of said presence being indicative for the presence of telomerase in the sample.

Optionally between steps (b) and (c) there is an additional step of providing a co-factor required for the nucleic acid catalytic activity. An example where the catalytic polynucleotide is DNAzyme and hemin and where it is ribozyme the co-factor may be  $Mg^{++}$ .

The inhibitory sequence is designed for example to produce a "hair pin" configuration with part of the catalytic nucleic acid sequence, such configuration (produced by hybridization of the catalytic polynucleotide and the inhibitory sequence) rendered the whole complex inactive.

The pre-catalytic complex also includes a primer for telomerase activity for example attached either to the free end of the catalytic polynucleotide or attached to the free end of the inhibitory sequence

. Production of a telomerase repeat sequence, cause the inhibitory sequence to hybridize with the newly formed repeat (instead of hybridizing with the catalytic nucleotide) thus "opening" the pre-catalytic complex and rendering it active (optionally together with a co-factor such as hemin).



## DESCRIPTION OF THE DRAWINGS

In order to understand the invention and to see how it may be carried out in practice, some preferred embodiments will now be described, by way of non-limiting examples only, with reference to the accompanying drawings, in which:

5       **Fig. 1** is the scheme for the analysis of telomerase activity using DNAzyme labels and chemiluminescence as a detection signal.

**Fig. 2** is the integrated light intensities corresponding to: (a) The analysis of 10,000 HeLa cells using the DNAzyme (7), 2.5  $\mu$ M. (b) The analysis of heat-treated HeLa cells (10,000) in the presence of the DNAzyme, 2.5  $\mu$ M. (c) The  
10      analysis of HeLa cells, 10,000 without the DNAzyme but upon interaction with hemin 2.5  $\mu$ M. (d) to (f) The analysis of 5000, 2500 and 1000 HeLa cells, respectively. Inset: Calibration curve corresponding to the analysis of variable numbers of cells.

**Fig 3** is the analyzing telomerase activity by a functional DNA beacon that  
15      self-generates a DNAzyme.

**Figure 4** Absorbance changes upon analyzing telomerase activity originating from: (a) 10,000 HeLa cells, (b) 10,000 heat-treated HeLa cells (95°C, 10 minutes). In all experiments the systems consisted of the catalytic beacon (5), 0.04  $\mu$ M, hemin, 0.04  $\mu$ M, ABTS, 3.2 mM and H<sub>2</sub>O<sub>2</sub>, 3.2 mM in 0.1 tris buffer  
20      solution, pH=8.1 that included MgCl<sub>2</sub>, 20 mM. Inset: Calibration curve corresponding to absorbance change of the system upon analyzing variable numbers of HeLa cells.



## DETAILED DESCRIPTION OF THE INVENTION

### I. EXPERIMENTAL PROCEDURES

#### A. Materials

5 Hemin was purchased from Porphyrin Products, (Logan, Utah), and used without further purification. The concentration of diluted hemin solutions was determined using standard spectroscopic methods.<sup>12</sup> A hemin stock solution was prepared in DMSO and stored in the dark at -20°C. Luminol and other chemicals were obtained from Sigma and used as supplied. All buffer solutions used in the  
10 different measurements contained the nonionic detergent Triton X-100 (0.05 % w/v) and DMSO (1 %, v/v).

DNA oligonucleotides were synthesized by Sigma Genosys. They were purified using the PAGE method. The sequences of the oligomers are given below:

- 15 (1): 5'-HS(CH<sub>2</sub>)<sub>6</sub>CGATTCGGTACTGG-3'  
(2): 5'-TTGAGCATGCGCATTATCTGAGCCAGTACCGAATCG-3'  
(3): 5'-ATGCGCATGCTCAATTTGGGTAGGGCGGGTTGGG-3'  
(5): 5'-HS(CH<sub>2</sub>)<sub>6</sub>TTTTTTAATCCGTCGAGCAGAGTT-3'  
(7): 5'-CTAACCCTAACCTTTGGGTAGGGCGGGTTGGG-3'

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#### B. Immobilization of the Thiolated DNA Primer and Hybridization of the DNAzyme Label

The Au-coated (50 nm gold layer) glass plate (22 mm × 11 mm) was pre-  
25 purified by the treatment with a piranha solution (consisting of 70 % concentrated sulfuric acid and 30 % hydrogen peroxide) for 20 min, and then thoroughly rinsed with pure water. The plate was then soaked in concentrated nitric acid for 5 min, and rinsed again with water. The plate was interacted with a solution of (1), 6 μM



in 0.4 M phosphate buffer, pH 7.4, for 12 h. The resulting plate was washed with the phosphate buffer and then the (1)-functionalized Au-surface was treated with 1-mercaptohexanol, 1 mM in 0.1 M phosphate buffer, pH 7.4, for 1 h. The resulting monolayer-functionalized surface was treated with different concentrations of the complementary analyte DNA (2) in a solution composed of 0.1 M phosphate buffer and the perfect Hyb<sup>TM</sup> plus hybridization buffer (Sigma) (1:1), for 5 h to yield the ds-DNA assembly on the surface.

A solution of 25  $\mu$ M of the DNAzyme (3) was heated at 95°C for 9 minutes in 0.01 M Tris buffer, pH 7.4, to dissociate any intermolecular quadruplex, and allowed to cool to room temperature. An identical volume of a buffer solution consisting of 50 mM HEPES, 40 mM KCl, 400 mM NaCl, 0.1 % Triton X-100 and 2 % DMSO, pH 7.4, was added to the (3) solution to allow appropriate folding. Hemin, 12  $\mu$ M, was added to the nucleic acid solution and the system was allowed to form the supramolecular complex for 3 h. The surface was then allowed to hybridize with 2.5  $\mu$ M solution of the DNAzyme in a 0.1 M phosphate buffer that included 25 mM HEPES, 20 mM KCl, 200 mM NaCl, 0.05 % Triton X-100 and 1 % DMSO, for 12 h.

### C. Preparation of Telomerase Extracts

HeLa cells were removed from the substrate by trypsinization, washed twice with PBS and pelleted at 2000 rpm for 10 min at 4°C. The cells were resuspended in a cold CHAPS lysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 0.1 mM PMSF, 0.5 % CHAPS (Sigma) and 10 % Glycerol) at a concentration of  $5 \times 10^6$  cells/mL, incubated for 30 min in ice and then centrifuged for 20 min (12,000 rpm, 4°C). The supernatant was flash frozen and stored at –70°C.

### D. Immobilization of (5) and telomerization on a Au-coated glass plate



The telomerase extract from the respective number of cells was introduced into 50  $\mu$ L of 20 mM Tris-HCl, pH 8.3, 4 mM  $MgCl_2$ , 1 mM EGTA, 63 mM KCl, 0.05 % Tween 20, 2 mM dATP, 2 mM dGTP and 2 mM dTTP. The reaction mixture, 50  $\mu$ L, was placed on the Au-coated glass plate modified with (5).  
5 Modification of the plate with (5) was performed as described for (1). The plate was covered and the telomerization was allowed to proceed for 12 h at 37°C. The resulting plate was rinsed with a phosphate buffer solution and allowed to hybridize with the DNAzyme solution, (7), 2.5  $\mu$ M, that was prepared as described for the analysis of (2). For the control experiments utilizing heat-treated HeLa cells, the  
10 cell extract was heated for 10 minutes at 85°C.

#### **E. Light Emission Measurements.**

Light emission was measured using a photon counting spectrometer (Edinburgh Instruments, FLS 920) equipped with a cooled photomultiplier  
15 detection system, connected to a computer (F900 v. 6.3 software). Before the samples analyses, a background run without sample was done, and all spectral results were corrected from the background and integrated. Measurements were made after the plates were placed in a cuvette that included 3.3 mL of a buffer solution consisting of 25 mM HEPES, 20 mM KCl and 200 mM NaCl, pH = 9.0,  
20 that included 0.5 mM luminol and 30 mM  $H_2O_2$ .

#### **EXAMPLE 1: DETECTION OF TELOMERASE ACTIVITY USING AMPLIFIED CHEMILUMINESCENCE SURFACE DETECTION**

Previous studies have indicated that a guanine-rich nucleic acid, with the  
25 base sequence depicted in Figure 1, structure I, is capable of forming a supramolecular G-quadruplex structure with hemin.<sup>6</sup> The resulting complex exhibited peroxidase-like catalytic activity, and it catalyzed the oxidation of 2,2'-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid, ABTS by  $H_2O_2$ . We find that this nucleic acid-hemin complex reveals also peroxidase-like functions towards the



oxidation of luminol by  $H_2O_2$  and the generation of chemiluminescence. We use this property to develop pre-designed DNAzyme label for the amplified detection of DNA.

Figure 1 depicts the method for the amplified analysis of telomerase activity. The primer (5) is assembled on a Au-surface, and the functionalized surface is interacted with the HeLa cancer cell extract in the presence of the nucleotide mixture dNTPs. Since the telomerization leads to a long nucleic acid with constant repeat units (6), the interface may be hybridized with a complementary catalytic label. The nucleic acid (7) is pre-designed to include the G-rich sequence that forms the catalytic complex with hemin, and a nucleic acid domain that is complementary to the telomere repeat units. The hybridization of the catalytic DNAzyme label with the telomere associated with the surface, enables then the chemiluminescence detection of the telomerase activity by the biocatalytic oxidation of luminol by  $H_2O_2$ , and the concomitant light emission. The analysis of telomerase involves two consecutive amplification steps. The first step involves the hybridization of several catalytic entities to the telomere and the second includes the catalytic DNAzyme that generates numerous photons as a result of a single telomere formation.

The system assembled on the gold surface was characterized by quartz crystal microbalance experiments. The immobilization of (5) on the Au/quartz surface resulted in a frequency change of -40 Hz that translates to a surface coverage of  $7.9 \times 10^{-12}$  mole-cm<sup>-2</sup> of (5). The telomerization occurring upon the treatment of the functionalized surface with a cell lysate (10,000 cells) in the presence of dNTPs leads to a frequency decrease of 52 Hz, and this translates to a coverage of  $6.9 \times 10^{-11}$  telomere units-cm<sup>-2</sup>. That is, an average ca. 9 telomere units are linked to each primer associated with the surface. (this frequency change corresponds to the incorporation of 54 bases into each primer linked to the electrode). The association of the catalytic (7)/hemin label with the surface further decreases the crystal frequency by 50 Hz, indicating a surface coverage of ca.



$1.3 \times 10^{-11}$  mole-cm<sup>-2</sup> or the binding of ca. two DNAzyme units to each telomeric primer.

Figure 2, curve (a), shows the integrated light intensity emitted from the system upon analyzing the telomerase activity originated from 10,000 cells. A control experiment revealed that upon analysis of heat-treated HeLa cells (85°C for 10 min) according to Figure 1, no light emission is observed, Figure 2, curve (b). That is, the thermal deactivation of the telomerase in the HeLa cells prevents telomerization, and the subsequent hybridization of (7) and the biocatalyzed generation of chemiluminescence is inhibited.

Furthermore, this control experiment demonstrates the advantages and utility of the DNAzyme as a label for the amplified detection of DNA. The fact that no chemiluminescence is generated by the heat-treated cells implies that no non-specific interference takes place in the system. Thus, even if cell ingredients bind non-specifically to the surface, their affinity to the catalytic DNAzyme is negligible. Also, the treatment of the telomere units on the surface with hemin leads only to a negligible generation of chemiluminescence, Figure 2, curve (c). Thus, although the telomere units includes G-bases, no biocatalytic complex is generated with hemin, and only the base sequence of (7) is specific to generate with hemin the DNAzyme of peroxidase activity. As the telomerization is controlled by the content of telomerase in the cell lysate samples, the amount of hybridized DNAzyme label, and the intensity of emitted light, should relate to the concentration of cancer cells. Figure 4 shows the integrated light intensity emitted from the system analyzing variable numbers of HeLa cells (curves (d) to (f)). As expected, the chemiluminescence decreases as the content of HeLa cells in the sample is lower. Figure 2, inset, shows the calibration curve that corresponds to the emitted light intensity as a function of the number of cells that are analyzed. The detection limit in this experiment corresponds to ca. 1000 HeLa cells in the analyzed sample.



## EXAMPLE 2: DETECTION OF TELOMERASES IN A SOLUBLE ASSAY

Scheme 3 depicts the method for the application of the beacon (soluble element) (1) as a catalytic unit for the sensing of DNA (2).

Fig. 3 depicts the method to analyze the telomerase activity by means of a catalytic beacon (soluble components). The beacon, (5), is designed to include at its two termini two functional nucleic acid components. One end of the hairpin structure is ends with a nucleic acid that includes the base sequence that is a part of the DNAzyme in the presence of hemin (part A). The second part of the DNAzyme base sequence (part B) is "hidden" in the hybridized hairpin configuration by the inhibitory sequence. At the other end of the hairpin, a nucleic acid segment that is a primer (6) for telomerase, and for the initiation of the telomerization, is tethered to the beacon (part C of the beacon). The single stranded loop of the beacon is complementary to the telomere repeat sequences. Treatment of the beacons with HeLa cancer cell extract in the presence of the dNTP nucleotide mixture, results in the telomerization of the hairpin end. The elongated telomere self-generates the sequence for its hybridization with the complementary hairpin loop (by the inhibitory sequence), and leads to the beacon opening, and to the generation of the DNAzyme. Thus, the telomerase activity is monitored by following the ABTS oxidation by  $H_2O_2$  upon the hairpin structure opening. Figure 4, curve (a), shows the time-dependent accumulation of the colored product (4) upon analyzing telomerase originating from 10,000 cells.

Figure 4, curve (b), shows the results of the control experiment where the accumulation of (4) from a system that included heat-treated ( $95^{\circ}C$  for 10 minutes) of 10,000 HeLa cell extract (the telomerase in the cells is deactivated upon heating). Clearly, the DNAzyme is not formed, and no color of (4) is developed in the system. The rate of the telomeres' formation is controlled by the content of telomerase in the sample, and thus the accumulation of (4) is regulated by the number of HeLa cells that are analyzed. Figure 2, insert, shows the absorbance values of (4), obtained upon the analysis of the telomerase activity originating from



– 13 –

different numbers of HeLa cells. (The absorbance of generated (4) is determined after a fixed time of telomerization corresponding to 8 minutes).



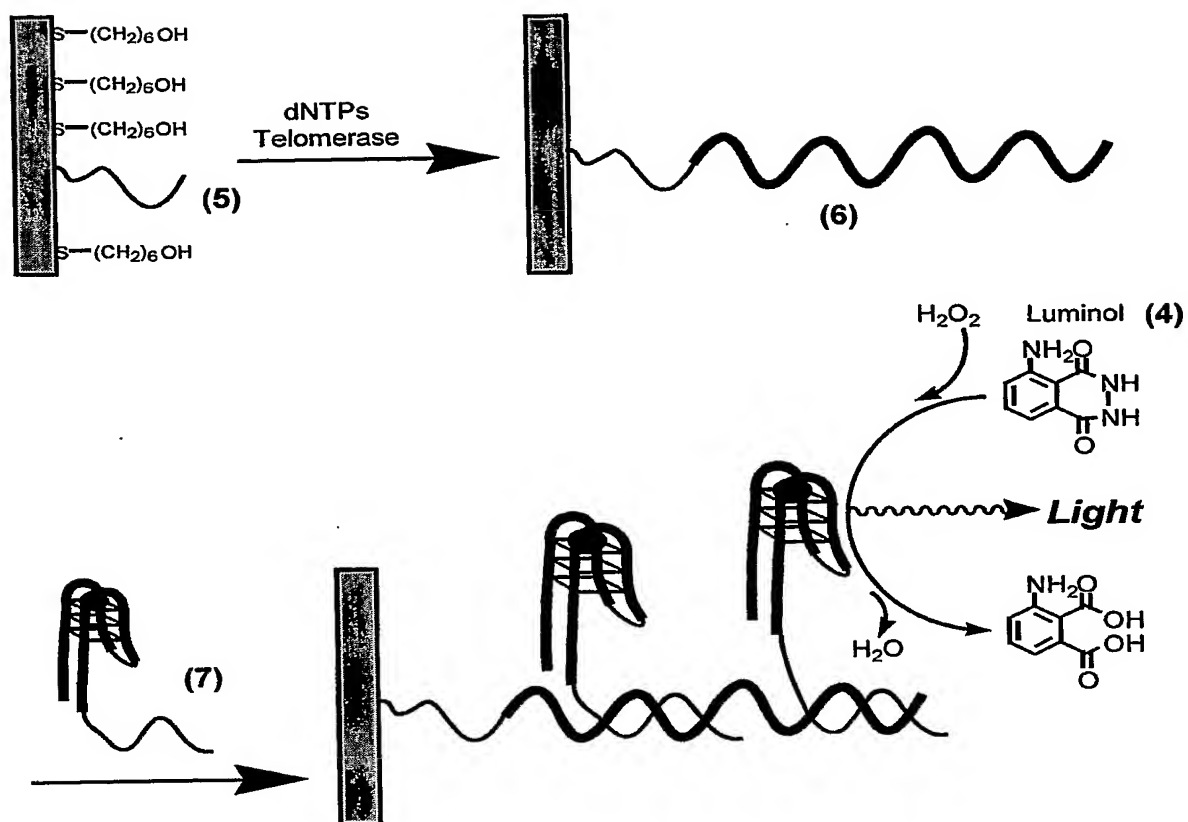
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(5) = 5'-HS(CH<sub>2</sub>)<sub>6</sub>TTTTTTAATCCGTCGAGCAGAGTT-3'  
 (6) = 5'-(GGTTAG)<sub>n</sub>-3'  
 (7) = 5'-CTAACCCTAACCTTTGGGTAGGGCGGGTTGGG-3'

**Fig 1.**



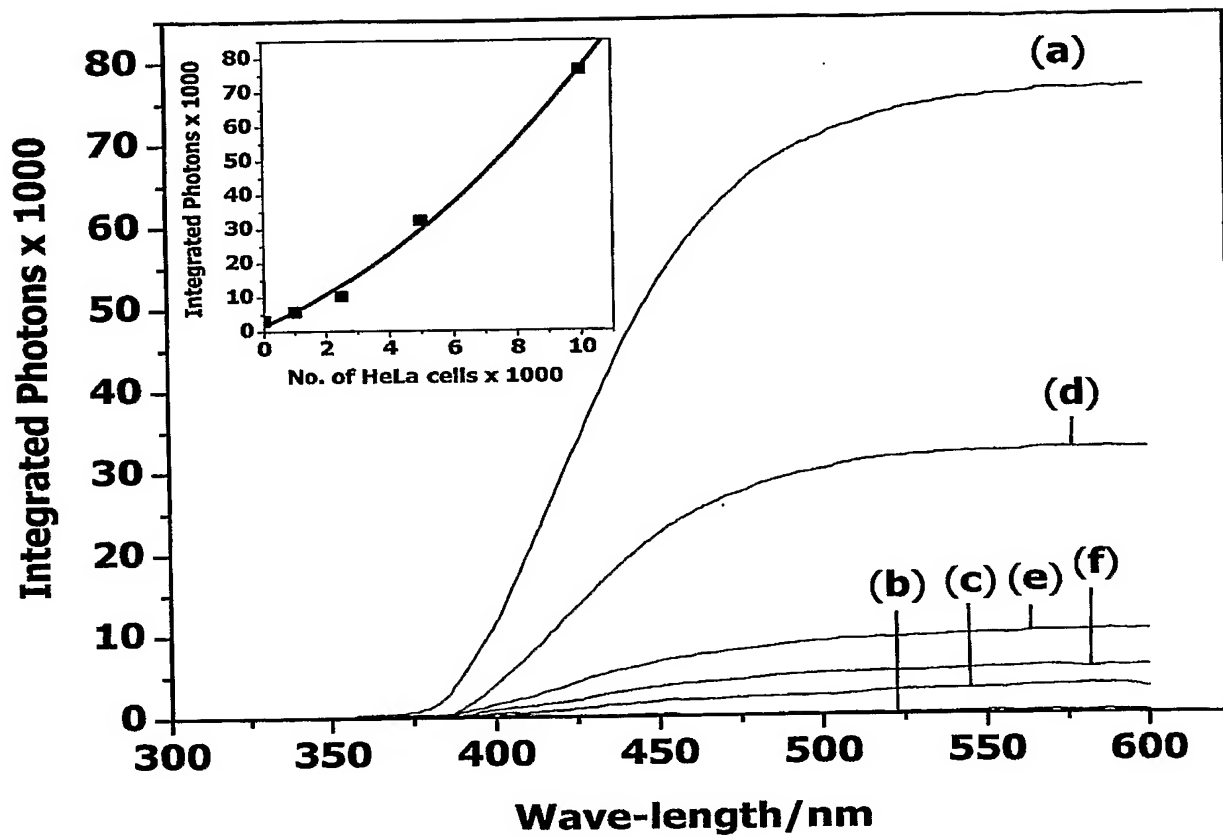
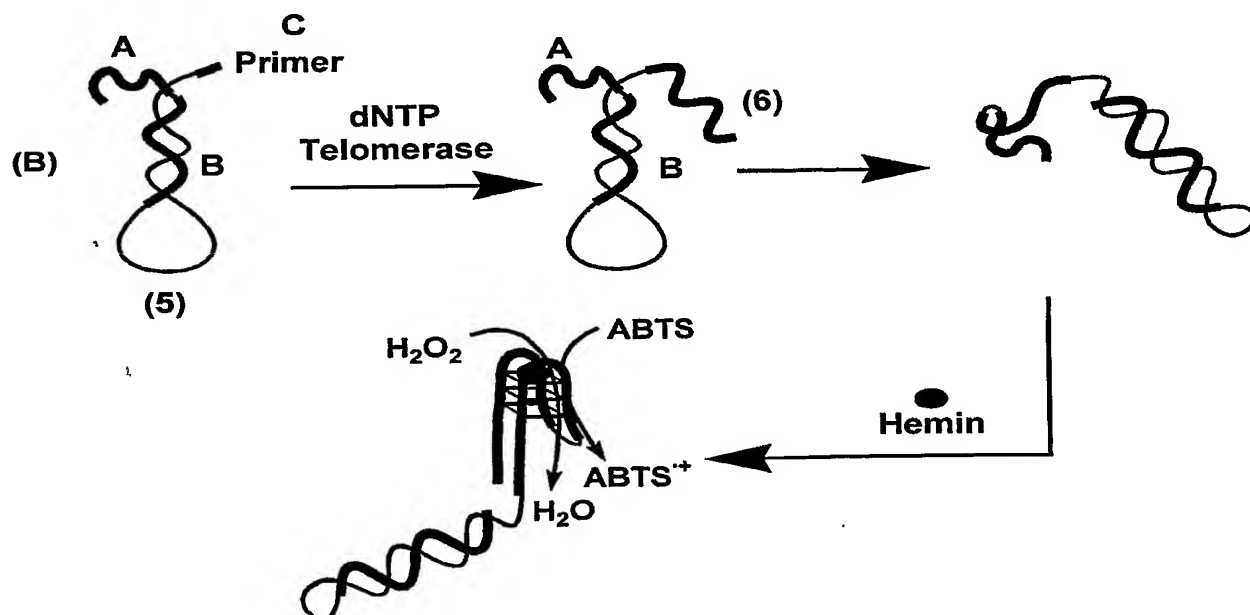


Fig 2



(1)=5'-CCCTACCCAGCCTTAAC TGTAGTACTGGTGAAATTGCTGCC  
ATTTGGGTAGGGCGGGTTGGG-3'  
(2) = 5'-AATGGCAGCAATTTCA C CAGTACTACAGTTAAGGC-3'  
(2a) = 5'-AATCGCAGCAATTTCA C CAGTACTACAGTTAAGGC-3'  
(2b) = 5'-AATGGCAGCAATTTCA C GAGTACTACAGTTAAGGC-3'



(5)=5'-TGGGTTAGGGCGGGTTGGGAAATAACCCCTAACCCCTAACCCCTAACCCCTAACCCCTAACCCCAACCCAATCCGTCGAGCAGAGTT -3'  
(6) = 5'-AATCCGTCGAGCAGAGTTAG(GGTTAG)n-3'

**FIG 3**



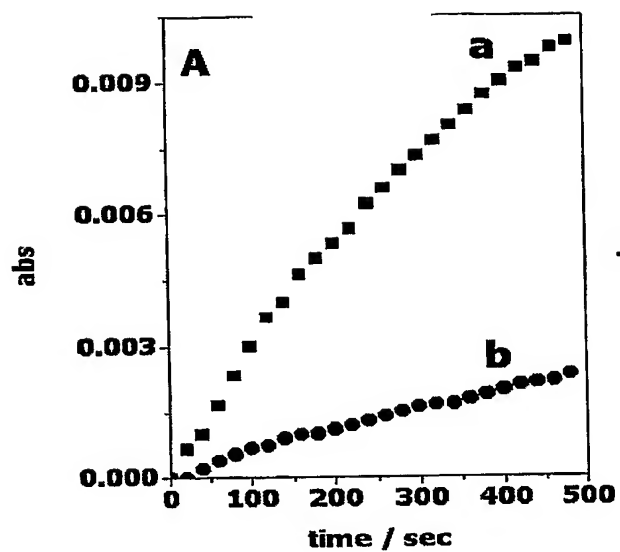


Fig. 4A

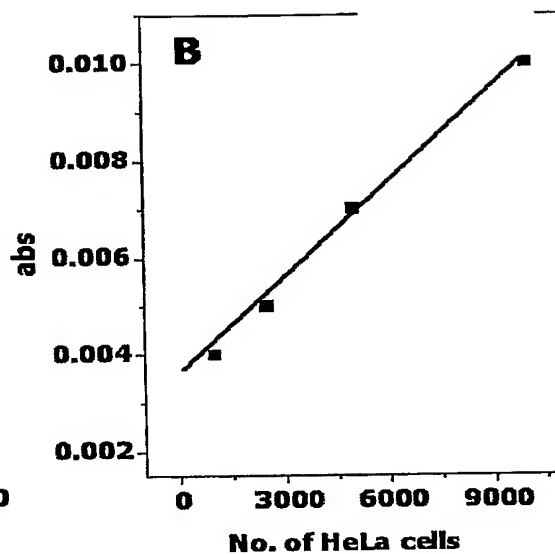


Fig. 4B